**Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A**

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**ABSTRACT**

Purified preparations of a distinct autophosphorylation-activated protein kinase from bovine kidney phosphorylated and inactivated purified preparations of protein phosphatase 2A (PP2A) by about 80% with the autophosphorylation-activated protein kinase, protamine kinase, and 32P-labeled myelin basic protein as substrates. Analysis of incubations performed in the presence of 0.2 mM [γ-32P]ATP by autoradiography following SDS/PAGE and by FPLC gel permeation chromatography on Superose 12 demonstrated that the catalytic subunit of PP2A was phosphorylated in the incubation mixtures containing the kinase and phosphatase. Up to 0.3 mol of phosphate groups was incorporated per mol of the catalytic subunit of PP2A following incubation with the kinase. This phosphorylation was enhanced about 5-fold in the presence of 0.4 μM microcystin-LR. In addition, up to 1 mol of phosphate groups was incorporated per mol of the PP2A subunit of apparent Mr = 60,000 when microcystin-LR was included. Analysis by thin-layer chromatography indicated that PP2A2 catalyzed an autodephosphorylation reaction which was inhibited by microcystin-LR. Phospho amino acid analysis showed that the catalytic subunit of PP2A2 was phosphorylated on threonine residues by the autophosphorylation-activated protein kinase. Together with previous observations, the results suggest that inactivation of PP2A by phosphorylation catalyzed by the autophosphorylation-activated protein kinase could contribute to the marked increase in the phosphorylation of cellular proteins in response to insulin and other mitogens.

**MATERIALS AND METHODS**

KC2 ethyl reverse-phase TLC plates were from Whatman. Silica-gel TLC plates were from Sigma. 32P, was from Amersham. Proline kinase (10) and PP2A (12) were purified to apparent homogeneity from extracts of bovine kidney as described. Other materials are given in refs. 9–15.

**Purification of Autophosphorylation-Activated Protein Kinase.** The kinase was purified as described (9). Bovine kidney cortex (2 kg) was homogenized in a Waring blender at high setting for 1 min with 2 vol of buffer A (25 mM Tris chloride, pH 7.0/1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/14 mM 2-mercaptoethanol). The homogenate was centrifuged for 30 min at 10,000 rpm in a Beckman JA-10 rotor and the pellets were discarded. To the supernatant was added with stirring 0.14 vol of 50% (wt/vol) poly(ethylene glycol). After 30 min, the mixture was centrifuged and the pellets were discarded. The supernatant was passed through glass wool and then applied onto a column (14 × 10 cm) of DEAE-cellulose equilibrated in buffer B (buffer A containing 10% (vol/vol) glycerol). The column was washed under suction with 4 liters of buffer B/0.05 M NaCl, and the combined effluent from DEAE-cellulose was then applied onto a column (5 × 6 cm) of poly(L-lysine)-agarose equilibrated in buffer B. The column was washed with 4 liters of buffer B and then developed with a 2000-ml linear gradient.

**Abbreviations:** PP2A, protein phosphatase 2A; MBP, myelin basic protein.

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of 0–0.5 M NaCl at a flow rate of 500 ml/hr, and 6.3-ml fractions were collected. Autophosphorylation-activated protein kinase was recovered at about 0.2 M NaCl. Active fractions were pooled, diluted with 4 vol of buffer B, and applied onto a column (2.5 × 6 cm) of heparin-agarose equilibrated with buffer B. The column was washed with 1 liter of buffer B and then developed with a 1000-ml linear gradient of 0–0.4 M NaCl at a flow rate of 300 ml/hr, and 6.3-ml fractions were collected. Autophosphorylation-activated protein kinase was recovered at about 0.2 M NaCl. The active fractions were pooled, mixed with 1 vol of buffer B containing 1 M NaCl, and then applied onto a column (2.5 × 4 cm) of phenyl-Sepharose equilibrated in buffer B/0.5 M NaCl. The column was washed with 500 ml of buffer B/0.25 M NaCl and then developed with a 600-ml linear gradient from buffer B/0.25 M NaCl to buffer B/65% (vol/vol) ethylene glycol/0.025 M NaCl/0.1% Triton X-100 at a flow rate of 90 ml/hr, and 3.8-ml fractions were collected. Autophosphorylation-activated protein kinase was recovered at about 30% ethylene glycol. The active fractions were pooled, mixed with 3 vol of buffer B and then applied onto a column (2.5 × 4 cm) of CM-Sepharose equilibrated in buffer B. The column was washed with 300 ml of buffer B and the combined effluent was applied onto a column (2.5 × 95 cm) of protamine-agarose equilibrated with buffer B. The column was washed with 500 ml of buffer B and then developed with a 600-ml linear gradient of 0–0.9 M NaCl at a flow rate of 300 ml/hr, and 5-ml fractions were collected. Autophosphorylation-activated protein kinase was recovered at about 0.6 M NaCl. The active fractions were pooled, diluted with 10 vol of buffer B, and then applied onto a small column (1.5 × 3 cm) of heparin-agarose equilibrated with buffer B. The column was washed with 50 ml of this buffer, and the kinase was then eluted with buffer B/1 M NaCl. The active fractions were pooled (about 2.5 ml) and applied onto a column (2.5 × 95 cm) of Sephacryl S-200 equilibrated and developed with buffer B/0.2 M NaCl. Fractions (1.8 ml) were collected and the flow rate was 20 ml/hr. The active fractions from Sephacryl S-200 were pooled and applied onto a column (1.5 × 1.5 cm) of phenyl-Sepharose equilibrated with buffer B/0.25 M NaCl. The column was washed with 50 ml of this buffer, and the kinase was eluted with buffer B/65% ethylene glycol/0.1% Triton X-100. The active fractions were pooled, aliquoted, and stored at −70°C. These preparations consisted of a single polypeptide of apparent Mr ≈ 36,000 as determined by SDS/PAGE (16).

**Determination of Autophosphorylation-Activated Protein Kinase Activity.** A 5-μl sample of the kinase was mixed with 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol in the absence or presence of 1 mM MgCl2 and 0.2 mM ATP in a final volume of 50 μl. After 10 min at 30°C, a 5-μl aliquot of the incubation mixture was added to 45 μl of 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol/10 mM MgCl2/0.2 mM [γ-32P]ATP (1000 cpmp pmol) containing 250 μg of bovine serum albumin and 45 μg of MBP in a microcentrifuge tube. After 10 min at 30°C, 1 ml of 12% (wt/vol) trichloroacetic acid was added and the solution was centrifuged at 10,000 rpm for 2 min in a Fisher microcentrifuge. The supernatant was discarded and the pellet was washed four times with 1-ml portions of 12% trichloroacetic acid. Aqueous counting scintillant was added to the tube and the radioactivity was determined. The kinase sample was omitted from controls. One unit of autophosphorylation-activated protein kinase activity was equivalent to the amount of the enzyme that catalyzed the incorporation of 1 nmol of phosphate into MBP per min. To ensure linearity, the extent of incorporation was limited to <100 pmol.

Proteine kinase activity was determined as described (13) except that 0.4 μM microcystin-LR was included in the reaction mixtures. One unit of proteine kinase activity was equivalent to the amount of enzyme that incorporated 1 nmol of phosphate into protamine sulfate per min.

**Determination of Protein Phosphatase Activity.** 32P-labeled MBP was prepared by incubation of 1 mg of MBP in 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride, 14 mM 2-mercaptoethanol/0.2 mM [γ-32P]ATP/10 mM MgCl2 containing 12.5 units of proteine kinase in a final volume of 0.25 ml. After 30 min at 30°C, 1 ml of 10% trichloroacetic acid was added and the mixture was centrifuged for 2 min in a Fisher microcentrifuge. The supernatant was discarded and the pellet was washed eight times with 1-ml portions of 10% trichloroacetic acid followed by three 1-ml portions of 95% ethanol. The pellet was suspended in 2 ml of 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol. The suspension was stored in aliquots at −70°C.

The phosphatase assay mixtures (50 μl) contained 50 mM Tris chloride (pH 7.0), 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 14 mM 2-mercaptoethanol, 0.2 mg of bovine serum albumin, a 5-μl sample of PP2A2, and 5 μg of 32P-labeled MBP. Reactions were initiated by the addition of the 32P-labeled substrate. After a 5-min incubation, 0.1 ml of 12% trichloroacetic acid was added and the mixture was centrifuged at 10,000 rpm in a Fisher microcentrifuge. An aliquot of the supernatant (0.12 ml) was mixed with 1 ml of scintillant and the radioactivity was determined. One unit of PP2A2 activity was equivalent to the amount of enzyme that released 1 nmol of 32P per min. To ensure linearity the extent of Pi release was limited to <10%.

SDS/12% PAGE was performed with the discontinuous buffer system of Laemmli (16). Radiolabeled bands were detected with Kodak X-Omat AR-5 film. Protein was determined by the procedure of Bradford (17).

**RESULTS**

Autophosphorylation-Activated Protein Kinase Inactivates PP2A2. During studies on the inactivation of autophosphorylation-activated protein kinase by PP2A2, we noted that when the incubation mixtures contained MgCl2 and ATP, the rate of inactivation of autophosphorylation-activated protein kinase was markedly reduced (Fig. 1). In contrast, when the incubation mixtures contained AMP, ADP, adenosine 5′-β,γ-imidodiphosphate, or GTP instead of ATP, little or no effect on the rate of inactivation of the kinase by the PP2A2 preparations was detected (Fig. 1). Incubation with autophosphorylation-activated protein kinase also inactivated the PP2A2 preparations in a time-dependent manner with 32P-labeled MBP or purified proteine kinase as substrates (Fig. 2).

Inactivation of PP2A2 preparations depended on the relative concentrations of PP2A2 and autophosphorylation-activated protein kinase. After 30 min at 30°C in the presence of 1 mM Mg2+ 0.2 mM ATP, and 0.1 μg of autophosphorylation-activated protein kinase, maximal inactivation (80–90%) of the PP2A2 preparations with 32P-labeled MBP as substrate was observed between 0.03 and 0.66 μg of PP2A2 and declined in the presence of 1.60 μg of the phosphatase. In the presence of 0.07 μg of PP2A2, half-maximal inactivation was observed at about 0.01 μg of the kinase. The optimal concentration for Mg2+ was 1–10 mM. At 1 mM Mg2+, the apparent Km for ATP was 20 μM. The optimal pH was about 7.0.

Autophosphorylation-Activated Protein Kinase Phosphorylates PP2A2. When autophosphorylation-activated protein kinase and PP2A2 were incubated with [γ-32P]ATP, SDS/PAGE followed by autoradiography showed that a protein of
FIG. 1. Effect of nucleotides on the PP2A-mediated inactivation of autophosphorylation-activated protein kinase. Autophosphorylation-activated protein kinase (50 ng) was activated in the presence of 0.2 mM ATP and 1 mM MgCl₂ in 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol in a final volume of 50 μl. After 15 min at 30°C, a 5-μl aliquot of the mixture was incubated (final volume, 50 μl) in 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol/1 mM MgCl₂ in the presence of 0.33 μg of PP2A₂ with or without the indicated nucleotides (0.2 mM). After 30 min at 30°C, 5 μl of the incubation mixture was used to determine autophosphorylation-activated protein kinase activity. AMPPNP, adenosine 5′- O-(β,γ-imido)triphosphate.

APPENDIX

Apparent Mr ≈ 36,000 was phosphorylated (Fig. 3A). This phosphorylation correlated closely with the inactivation of the PP2A₂ preparations. However, because autophosphorylation-activated protein kinase and the catalytic subunit of

Fig. 2. Autophosphorylation-activated protein kinase inactivates PP2A₂. Autophosphorylation-activated protein kinase (50 ng) was incubated for 15 min at 30°C in the presence of 0.2 mM ATP and 1 mM MgCl₂ in 50 mM Tris chloride, pH 7.0/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol in a final volume of 35 μl. A 5-μl aliquot of PP2A₂ (0.33 μg) was then added to the incubations. At the indicated times, 5 μl of ³²P-labeled MBP (5 μg; ●) or protamine kinase (7.5 units; ▲) was added. After 3 min of incubation at 30°C, dephosphorylation of ³²P-labeled MBP was terminated with 100 μl of 12% trichloroacetic acid and phosphatase activity was determined. After 10 min of incubation, protamine kinase activity was determined as described (13), except that the reaction mixtures contained 0.4 μM microcystin-LR. Open symbols represent control incubations in which autophosphorylation-activated protein kinase was omitted.

PP2A₂ comigrate with an apparent Mr ≈ 36,000 in SDS/polyacrylamide gels, the incubations were analyzed further by FPLC gel permeation chromatography on a calibrated

Fig. 3. Autophosphorylation-activated protein kinase phosphorylates PP2A₂. Autophosphorylation-activated protein kinase (50 ng) was activated in the presence of 0.2 mM ATP and 1 mM Mg²⁺ as described in the legend to Fig. 1. An aliquot (2.5 μl) was then incubated at 30°C (final volume, 25 μl) in the presence of 1 mM Mg²⁺ and 0.2 mM [γ-³²P]ATP in the presence (lanes 1–4) or absence (lanes 5–8) of 0.33 μg of PP2A₂. PP2A₂ was also incubated in the absence of the kinase (lanes 9–12). At the indicated times, sample buffer (16) was added, the mixtures were heated at 100°C, and SDS/PAGE (16) was performed. The gel was stained with Coomassie blue, washed extensively, dried, and exposed to x-ray film. (A) Autoradiogram of the dried gel. Arrow denotes the position corresponding to the catalytic subunit of PP2A₂ (Mr ≈ 36,000). (B) Incubations with the kinase and phosphatase were scaled up 4-fold to a final volume of 0.1 ml and were performed as described above in the absence (●) or presence (○) of 0.4 μM microcystin-LR. After 20 min at 30°C, the mixtures were subjected separately to FPLC gel permeation chromatography on Superose 12 equilibrated and developed at a flow rate of 0.2 ml/min in 50 mM Tris chloride, pH 7.0/0.2 M NaCl/10% glycerol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol. Fractions (200 μl) were collected, and aliquots (25 μl) of the fractions were subjected to SDS/PAGE followed by electrophoretic transfer onto Immobilon-P transfer membranes (Millipore). The bands corresponding to the catalytic subunit of PP2A₂ were identified by staining with Ponceau S and excised from the membrane strips for liquid scintillation counting. Arrows denote the positions at which purified preparations of PP2A₂ and autophosphorylation-activated protein kinase (AK) were eluted from the column. Aliquots of the fractions exhibiting the highest radioactivity from the incubations performed in the absence (lane 1) or presence (lane 2) of microcystin-LR were then electrophoretically transferred onto Immobilon-P transfer membranes after SDS/PAGE. The membrane strips were stained with Ponceau S, washed with water, dried, and then exposed to x-ray film. Inset shows an autoradiogram of the dried membrane strips. Arrows denote the positions corresponding to the PP2A₂ subunits of apparent Mr ≈ 60,000 and 36,000.
subunit of apparent $M_r \approx 60,000$ (Fig. 3B). Control incubations in which PP2A$_2$ and autophosphorylation-activated protein kinase were incubated separately in the absence or presence of microcystin-LR showed little or no phosphorylation (data not shown). There was also little or no $^{32}$P-label associated with fractions containing autophosphorylation-activated kinase (Fig. 3). We estimate that up to 0.3 mol and 1.5 mol of phosphate was incorporated per mol of the catalytic subunit of the PP2A$_2$ preparations following incubation with autophosphorylation-activated kinase in the absence and presence of microcystin-LR, respectively. Phospho amino acid analysis indicated that the catalytic subunit of the PP2A$_2$ preparations was phosphorylated on threonine residues (Fig. 4).

**Autodephosphorylation of PP2A$_2$.** The results suggested that in the absence of microcystin-LR, PP2A$_2$ catalyzed an autodephosphorylation reaction. To examine this possibility, the incubation mixtures were analyzed by autoradiography. In the absence of microcystin-LR, phosphate was released in the incubation mixtures containing the kinase and phosphatase; whereas in the presence of microcystin-LR, this dephosphorylation was inhibited (Fig. 5).

**DISCUSSION**

The results demonstrate that PP2A$_2$ is inactivated by phosphorylation of its catalytic subunit following incubation with purified preparations of autophosphorylation-activated protein kinase. Thus, following incubation with purified preparations of autophosphorylation-activated protein kinase, PP2A$_2$ was inactivated by up to 80% with autophosphorylation-activated protein kinase (Fig. 1). $^{32}$P-labeled microcystin-LR (Fig. 2) and protamine kinase (Fig. 2) as substrates. Under these conditions, up to 0.3 mol of phosphate was incorporated per mol of the catalytic subunit of PP2A$_2$ (Fig. 3). Phospho amino acid analysis indicated that the catalytic subunit of PP2A$_2$ was phosphorylated on threonine residues (Fig. 4). The location of the phosphorylation site(s) remains to be determined.

Analysis by TLC indicated that PP2A$_2$ catalyzed an autodephosphorylation reaction during incubation with the kinase (Fig. 5). This dephosphorylation was inhibited when the incubations were performed in the presence of the PP2A inhibitor microcystin-LR (Fig. 5). Moreover, in the presence of microcystin-LR, the extent of phosphate incorporation into the catalytic subunit of PP2A$_2$ was enhanced 5-fold (Fig. 3). In addition, under these conditions up to 1 mol of phosphate was incorporated per mol of the PP2A$_2$ subunit of apparent $M_r \approx 60,000$ following incubation with the kinase (Fig. 3). Phosphorylation of this subunit was not detected when the incubations were performed in the absence of microcystin-LR (Fig. 3) and at all dilutions of the phosphatase (data not shown). These results indicate that phosphorylation of the catalytic subunit of the PP2A$_2$ preparations by autophosphorylation-activated protein kinase did not inactivate PP2A$_2$ with its own subunit of apparent $M_r \approx 60,000$. Because the specific activity of autodephosphorylation depended directly on the concentration of PP2A$_2$ employed in the incubations (data not shown), autodephosphorylation appears to occur via an intermolecular rather than intramolecular reaction mechanism. Therefore, we cannot entirely rule out the possibility that dephosphorylation was catalyzed by a contaminating phosphatase present in the incubation mixtures. Interestingly, Serra et al. (18) have reported the purification to apparent homogeneity of a protein of apparent $M_r \approx 20,000$ which inhibited the hydroxymethylglutaryl-CoA reductase phosphatase activity of purified preparations of PP2A$_2$ without affecting the phosphorylase phosphatase activity of this enzyme. Whether this and/or another physiological protein or effector inhibits the rate of this apparent autodephosphor-
ylation of PP2A remains to be determined. In addition, whether phosphorylation of PP2A by autophosphorylation-activated protein kinase affects the low but detectable protein-tyrosine-phosphatase activity of PP2A (19–21) also remains to be determined. It is pertinent that this protein-tyrosine-phosphatase activity of PP2A was stimulated by purified preparations of an ATP- and Mg²⁺-dependent protein factor from Xenopus oocytes (22). However, the mechanism of action of this activating protein factor is uncertain, and phosphorylation of PP2A does not appear to be involved (22).

Our results suggest that phosphorylation and inactivation of PP2A may be a physiologic mechanism by which auto-phosphorylation and activation of autophosphorylation-activated protein kinase and phosphorylation and activation of the protamine kinase may occur in cells in response to extracellular stimuli. It is important to note that the activity of the protamine kinase is stimulated rapidly following incubation of isolated rat hepatocytes with insulin (11), apparently via phosphorylation of the kinase (11). In addition, the evidence indicates that PP2A is a specific protamine kinase-inactivating phosphatase (12). Thus, in contrast to the other three major cytoplasmic protein phosphatase (PP1, PP2B, and PP2C), PP2A specifically inactivated purified preparations of the protamine kinase (12). Interestingly, relative to PP1, PP2A preferentially inactivated mitogen-activated protein kinase (23), two distinct mitogen-activated ribosomal protein S6 kinases (23, 24), and an insulin-stimulated Kemptide kinase (25). Thus, it is also possible that inactivation of PP2A by phosphorylation may contribute to the phosphorylation and activation of these insulin- and mitogen-activated protein kinases also and thereby contribute to the marked increase in the phosphorylation of cellular proteins in response to insulin and other mitogens.

However, in addition to its activity with the insulin- and mitogen-regulated protein kinases, PP2A exhibits activity with numerous proteins that are also phosphorylated in response to insulin and other mitogens. For example, acetyl-CoA carboxylase (26), ATP-citrate lyase (26), and the mRNA cap-binding protein, initiation factor 4E (27, 28) are phosphorylated in intact cells in response to insulin and other mitogens. Acetyl-CoA carboxylase and ATP-citrate lyase are some of the best PP2A substrates identified to date (1), and PP2A is an initiation factor 4E phosphatase (G. D. Amick and Z.D., unpublished data). Thus, phosphorylation and inactivation of PP2A in response to insulin and other mitogens may also contribute to the phosphorylation of these proteins. Previous failures to observe an effect of insulin and other mitogens on PP2A activity (29, 30) may well be because the conditions employed did not prevent the dephosphorylation of PP2A either by autodephosphorylation or by other PP2A phosphatases present in extracts from the control and hormone-treated cells.

While this manuscript was in the final stages of preparation, Brautigan and coworkers (31) reported that purified preparations of the catalytic subunit of PP2A could be phosphorylated transiently on tyrosine residues by the tyrosine-specific insulin and epidermal growth factor receptor kinases and by the non-receptor tyrosine-specific kinases p60⁶⁻⁺ and p56ck. The effects of the p56ck- and insulin receptor-', and epidermal growth factor receptor-catalyzed phosphorylation on PP2A activity were not determined. However, thio phosphorylation by p60⁶⁻⁺ purified by immunoprecipitation from cells overexpressing the gene for this enzyme was reported to inactivate the purified preparations of the catalytic subunit of PP2A with phosphorylase as substrate (31). These results are in contrast to those reported in this paper, which show that PP2A₂ is inactivated by phosphorylation of its catalytic subunit on threonines catalyzed by purified preparations of the autophosphorylation-activated protein kinase. It is nevertheless possible that in intact cells multiphasic phosphorylation of the catalytic subunit of PP2A on threonine and tyrosine residues may, in response to extracellular stimuli, be a mechanism for "locking for the duration" the phosphorylated and inactivated form of PP2A.

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